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14. ABSTRACT Our original proposal requested a PMI Capillary Flow Porometer (CFP) to measure porosity, pore size and volume distribution, interconnectivity, through pore surface area, and permeability on small and thin porous media. We proposed that this was a challenging, but critical, aspect in studying functional materials for various applications, including biological/chemical sensors, bio-fuel cells and batteries.						
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SUMMARY

The PI is currently funded through a subaward agreement with the University of New Mexico under an Air Force MURI award titled Fundamentals and Bioengineering of Enzymatic Fuel Cells (P. Atanasov, PI). His initial role in the MURI interdisciplinary effort was to investigate the direct and *in-situ* determination of immobilized enzyme activity and loading. This role has since expanded to include the fabrication of macroporous scaffolds and the characterization of their pore structure. To fully exploit these measurements, however, knowledge of the permeability and mean pore structure were deemed important. A key element in this measurement is the detailed quantification of electrode properties such as porosity, pore size distribution, pore channel diameter and pore volume distribution, effective surface area, and tortuosity. When coupled with measurement of the total enzyme loading and spatial distribution, as well as spectro-electrochemical analysis, detailed characterization of electrochemical performance of enzyme-modified electrodes is obtainable.

Our original proposal requested a PMI Capillary Flow Porometer (CFP) to measure porosity, pore size and volume distribution, interconnectivity, through-pore surface area, and permeability on small and thin porous media. We proposed that this was a challenging, but critical, aspect in studying functional materials for various applications, including biological/chemical sensors, bio-fuel cells and batteries. The CFP was requested to yield data that helped characterize the linkage of total enzyme loading and its spatial distribution to the envelope surface area, under the assumption that this correlation would permit a more comprehensive analysis of the charge transfer efficiency in enzyme catalyzed electrodes.

After extensive and unsuccessful efforts were made to obtain preliminary analysis of our samples from PMI, and after some discussion of PMI's instruments and surface engineering with prior customers who expressed, confidentially, dissatisfaction with the product, we concluded that PMI was either unable to provide an instrument that would work on our sample, or no longer able to provide serviceable instruments. Regardless, without prior sample analysis a purchase for this instrument could not be managed through purchasing. With this reality the PI began an extensive search of instrumentation that could analyze pore structure. We selected the Keyence 3D laser scanning confocal microscope, which we felt provided the greatest benefit to our project, and the greatest additional benefit and synergy to the our MURI partners. In summary, this decision, which was made in consultation with the program manager, Major Jennifer Gresham, was made after (1) efforts to obtain preliminary data with PMI were unsuccessful, (2) extensive review of other similar analytical instrumentation (e.g., mercury porosimetry), and (3) the opportunities for pore structure and surface topology measurements with the Keyence 3D laser scanning microscope became evident. The rational for this decision is now outlined and preliminary results presented.

RATIONAL

There is no singular method to analyze pore structure, nor is there one way to characterize pore structure. Consequently, there are a number of instruments on the market that can be used to analyze pore structure, most of which approach the measurement differently and yield different

information. A good summary can be found in a recent publication titled "A comparison of micro CT with other techniques used in the characterization of scaffolds"¹. This article analyzed the value of six techniques used to characterize pore structure: Scanning electron microscopy, mercury porosimetry, gas pycnometry, gas adsorption, PMI's flow porosimetry, and Micro CT. On page 1374 of this article the authors summarize the relative properties each measurement technique provides (or does not). The study concluded the inability of each technique to provide the complete suite of commonly accepted characterization measurements, including PMI's flow porosimeters. And while some instrumentation may have overlapped on some measurements (e.g. mercury and flow porosimetry), other instrumentation was radically different (e.g., Micro CT).

Consequently, it was sought to select an instrument that (1) provided pore structure analyses that cannot easily be obtained through contract hire or collaboration with mainland researchers, (2) provided the type of measurements that are versatile in terms of their usefulness to other research applications (i.e., added value as a lab instrument), (3) provided the type of pore structure analyses that are most required on a regular or semi-regular basis, (4) would last over time with minimal requirements in terms of cost of maintenance and upkeep, and (5) minimized environmental hazards and hazardous waste disposal.

The requested Keyence Color 3D laser scanning microscope was judged to best fit this criteria. The Keyence microscope, which was not reviewed in the referenced article because of its newness to the marketplace, bears some similarity to the scanning electron microscope (SEM). The Keyence is a surface imaging microscope that provides high resolution images. The Keyence microscope, unlike the SEM, provides the following features: (1) it does not require pre-processing of the sample with the deposition of gold along the surface, (2) it does not destroy any part of the sample (which can sometimes happen at very high magnification in the SEM), (3) it does not require the application of vacuum surrounding the sample, (4) it can handle much larger sample sizes and therefore may not require breaking the sample up into smaller parts, (5) it can provide color images in real time without post measurement processing using alternative software, and (6) it can provide 3D measurement and analysis of the surface profile in situ and in real time while the sample is being viewed under high magnification (this permits one to immediately measure with numerical precision the height and depths of protrusions or holes along the surface).

Perhaps more than any single of these essential features possessed by the Keyence microscope is its ability to analyze the 3D surface structure with numerical precision. This means that unlike our current work with the SEM, whereby one must analyze pore structure by first taking images and then analyzing those images with software that estimates the pore diameters, the Keyence provides software that not only measure the diameter of surface pores, but also their depth. This feature really combines both traditional SEM measurements with surface roughness measurements, thus permitting (at resolutions equal to or even exceeding their counterparts) to profile surface structures in terms of their shape, roughness, surface area and volume, as well as profile comparisons between samples. More, this measurement can be done rapidly, within

¹ Saey Tuan Ho and Dietmar W. Hutmacher (2006). *A comparison of micro CT with other techniques used in the characterization of scaffolds*, *Biomaterials*, 27(8): 1362-1376.

minutes (as compared to roughly 30 minutes to 1 hour per sample with an SEM) and with no required sample preparation (i.e. the sample is simply put on the sample stage just like an optical microscope). The measurement is also totally non destructive to the sample (this is a very attractive feature in that it allows us to then use this sample in other characterization instruments).

There are some added features that make the Keyence very attractive (relative to many of the other instruments), and therefore worth considering. First, the Keyence microscope can also serve as an optical microscope. This means that in addition to resolution of surface features in 3D down to dimensions of 150 nanometers, the Keyence microscope can also be used for traditional optical viewing of samples at very high magnification. Second, the Keyence microscope is very low maintenance in that the only moving part (besides the sample table) is the laser (and this really doesn't move). This avoids an expensive service contract. Third, the Keyence microscope does not require the application of a vacuum (like scanning electron microscopes), or use low volume high pressure pumps that must be intricately controlled (as in required in all porosimeters). This avoids destruction of the sample. Fourth, the Keyence microscope does not consume or produce hazardous materials (as does mercury porosimeters). This avoids expensive hazardous waste disposal costs.

EQUIPMENT REVIEW

In the course of my evaluation of instrumentation for the analysis of pore structure, I considered (1) flow porosimeters from PMI Inc., (2) the Automated Standard Porosimeter from Porotech Ltd, (3) the Mercury Porosimeter from Micromeritics Instrument Corp., (4) the X-Ray CT scanner from Microphotronics, and (5) The LEXT OLS-3000IR Infra Red Confocal Microscope. Each of these are now reviewed in the context of evaluating their effectiveness to meet the objectives of the DURIP grant and University purchasing policies.

1: PMI's flow porosimeters.

PMI's flow porosimeter were originally considered for our application because of their promise to obtain pore structure analysis of the entire sample, and in reference to the direction of liquid flow through the sample. This novel feature promised novel information on material permeability in the direction of fluid flow. However, in the course of pursuing pre-purchase analysis of a sample, I encountered several troubling issues. First, PMI never analyzed my sample despite repeated emails and phone calls with the President (Dr. Krishna Gupta), the testing lab, and office staff. I also came across the name of a previous customer who had purchased a PMI instrument in 2004, never used it, and would not speak further on the subject due to potential legal issues (Ms. Tamary Nicholson). In fairness, I asked PMI to provide me with four customer references. The manager at PMI (Ms. Jaquiline West) declined to give out references, citing confidentiality. As this is not a standard operating practice, I concluded that PMI could not provide an instrument that was durable, easy to operate, reliable, and supported by an engineering staff (even if a service contract was required). For these reasons I eliminated any instrumentation purchase from PMI Inc.

2: Porotech's Automated Standard Porosimeter

Porotech's instrument uses the Method of Standard Porosimetry (MSP) to investigate any type of porous material, including soft, frail, materials prone to amalgamation, and powders. The method is relatively simple and nondestructive it enables measurements in the widest range of pore sizes. MSP can also be used for evaluation of the contact angle as well as hydrophilic-hydrophobic properties of multi-component materials. Thus, MSP enables studying of very different porous materials and specimens in their real environment. MSP does not require high pressures and is environmentally friendly. Although some of the general type of pore structure analyses returned (pore volume distribution, average pore radius, adsorption isotherms) are generally similar to PMI's various instruments, the Automated Standard Porosimeter also promises the unique advantage of using hydrophobic and hydrophilic wetting fluids. This feature permits one to look at liquid distribution of fluids within the pores as a function of the free binding energy between the liquid and the material.

While promising, the PI was also unable to have my sample analyzed prior to the purchase. Despite sending them a sample, and repeated requests, no analysis was ever completed, in part because this instrument requires that the sample be dried under vacuum at elevated temperature to fully remove any water or retained fluid. As our samples are made of porous chitosan, a biocompatible polysaccharide, they are both fragile and subject to decomposition at 80°C it was suspected that our samples could not be prepared for analysis by this instrument. Finally, the sample size required by this analysis is much larger than the typical sample size we prepare in the lab, and this makes it problematic. For these reasons, as well as the realization that these types of pore structure analyses provided by PMI and Porotech, can be purchased on a sample by sample basis by larger companies that produce well established mercury porosimeters (see 3 below), Porotech's Automated Standard Porosimeter was eliminated.

3: Micromeritics' Mercury Porosimeter.

Micromeritics is a large company and long time producer of porosimeters using mercury. This technique, which performs nearly all the analyses offered by PMI and Porotech's instruments, is considered the classic technology in the characterization of pore size distribution in porous materials. The major limitation to mercury porosimeters, despite its obvious compatibility with our requirements, is the fact that it uses mercury. While one of our samples was very well analyzed by Micromeritics using a mercury porosimeter, the restrictions of using mercury are were too difficult to justify from University Environmental Health and Safety standards. Bearing in mind that each and every sample analyzed is considered a hazardous waste that requires stringent disposal methods, and costly disposal, the PI was highly discouraged to purchase this instrument. In addition, the mercury in the porosimeter must be periodically shipped to a mainland site to be cleaned of contaminants. For these reasons, and the fact that the measurements provided by the instrument can be purchased on pay for use basis, this instrument was eliminated.

4: Microphotonic's Micro CT.

The Microphotonic's Micro CT is an X-ray machine that can scan a sample and provide a 3D image of the sample structure. This instrument provides excellent 3D pore structure analysis because of its ability to provide X-Y images of the sample in 5 micron slices and thus provides a truly non invasive way to "image" the pore structure in 3D. Using software provided with the instrument, 3D images can be remade from the sliced images, much like is done by software in

laser scanning microscopes. More, because the each sliced image can be analyzed one by one, the software also provides the ability to characterize the degree of interconnectivity between the pores, a very important measurement characterizing 3D pore structure. Unfortunately, analysis of our sample using the only instrument that could be purchased for \$100K was not powerful enough to resolve the details of our highly porous samples. As the pre-purchase analyses of these samples were unable to resolve any features of the material, this instrument was deemed unsuitable for our purposes. For this reasons the Micro CT scanner was eliminated.

5: The LEXT OLS-3000IR Infra Red Confocal Microscope

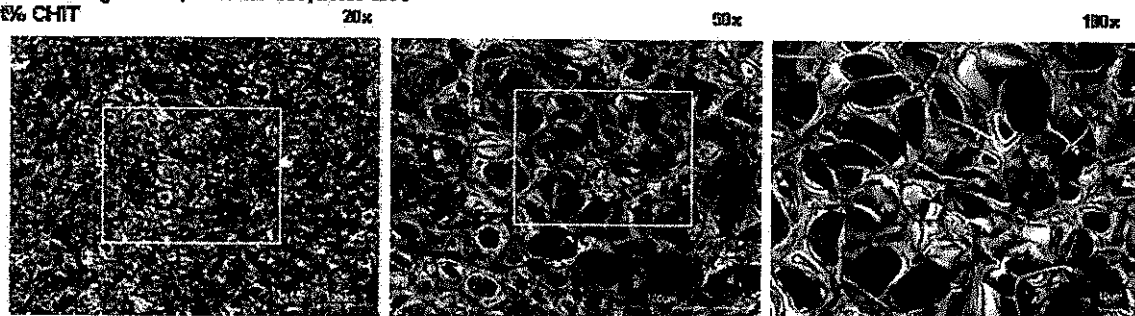
The LEXT OLS-3000IR Infra Red Confocal Microscope is Olympus's new near-IR laser based confocal microscope for nondestructive interior observation of silicon wafers, IC chips, MEMS and other devices. The Infra Red Confocal Microscope - LEXT IR enables features that cannot be seen visually -- such as SIP (System in Package), 3-dimensional mounting, and CSP (Chip Scale Package) -- to be inspected, measured and analyzed nondestructively. This microscope is similar to the Keyence microscope in that it uses confocal laser scanning technology to deliver three dimensional images. However, the LEXT-IR requires material to be present to report back an image because it requires that the material being exposed to the near infrared laser emit a response signal. While this works well for highlighting internal fractures below the surface of solid state materials like silicon wafers, or even along the surface, the technology was mostly unable to analyze our chitosan samples which, again, are very porous (over 97%) are almost invisible to the LEXT-IR. As such, virtually no features could be obtained that would permit any post process software imaging and analysis of the pore structure, along the surface or at any height below the surface. For this reason this instrument was rejected.

RESULTS

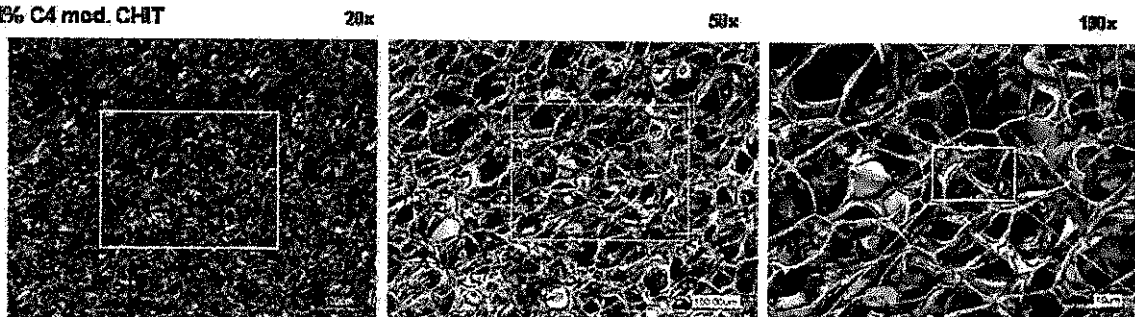
Contribution to the enhancement of research and research related education currently funded by the DoD

Measuring porosity, pore size and volume distribution, interconnectivity, through-pore surface area, and permeability on small and thin porous media is a challenging, but critical, aspect in studying functional materials for various practical applications, including biological / chemical sensors and bio-fuel cells. The PI is currently funded through a subaward agreement with the University of New Mexico (P. Atanassov, PI) under a MURI award titled Fundamentals and Bioengineering of Enzymatic Fuel Cells. Our specific role in this interdisciplinary effort is the direct and *in-situ* characterization of immobilized enzyme loading and its correlation to electrode activity. This role has since expanded to include the fabrication of macroporous scaffolds and the characterization of their pore structure. They Keyence has already provided images that are being used to characterize the pore structure and surface topology of chitosan scaffolds. These images, provided in Figures 1 through 4 below, clearly demonstrate the clear resolution of pore structure down to 0.5 μm , as well as surface topography analysis.

03-12-2009 KEYENCE images C.L.
100 μ L scaffold on glass slide, frozen 1hr -20C, (cells dead)
1wt% CHIT



1wt% C4 mod. CHIT



500x



Figure 1

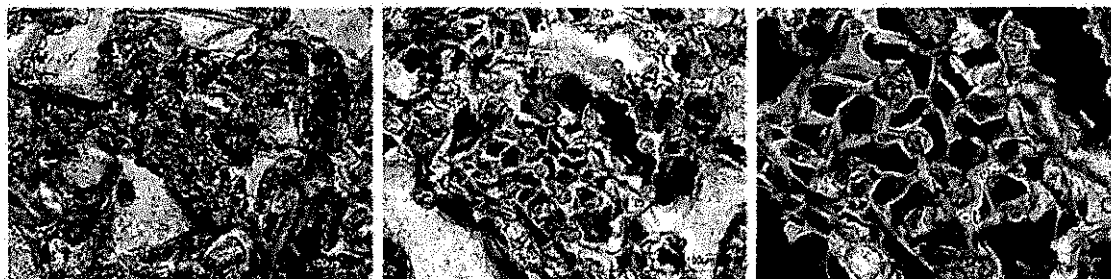
03-13-2008 KEYENCE Images C.L.
10 μ L scaffold on glass slide, frozen 1hr -20C, freeze dried

1wt% CHIT 5wt% CNT
080313_CHIT_CNT I

20x

50x

100x



1wt% C4 mod. CHIT 5wt% CNT
080314_C4_CNT pl

20x

50x

100x



Figure 2

03-12-2008 KEYENCE images C.L.

10ul scaffold on glass slide, frozen 1h/-20C, freeze dried

1wt% CHIT

Dry scaffold film

1 min after re-suspension in EtOH

10 min after re-suspension in EtOH (dry again)



Figure 3

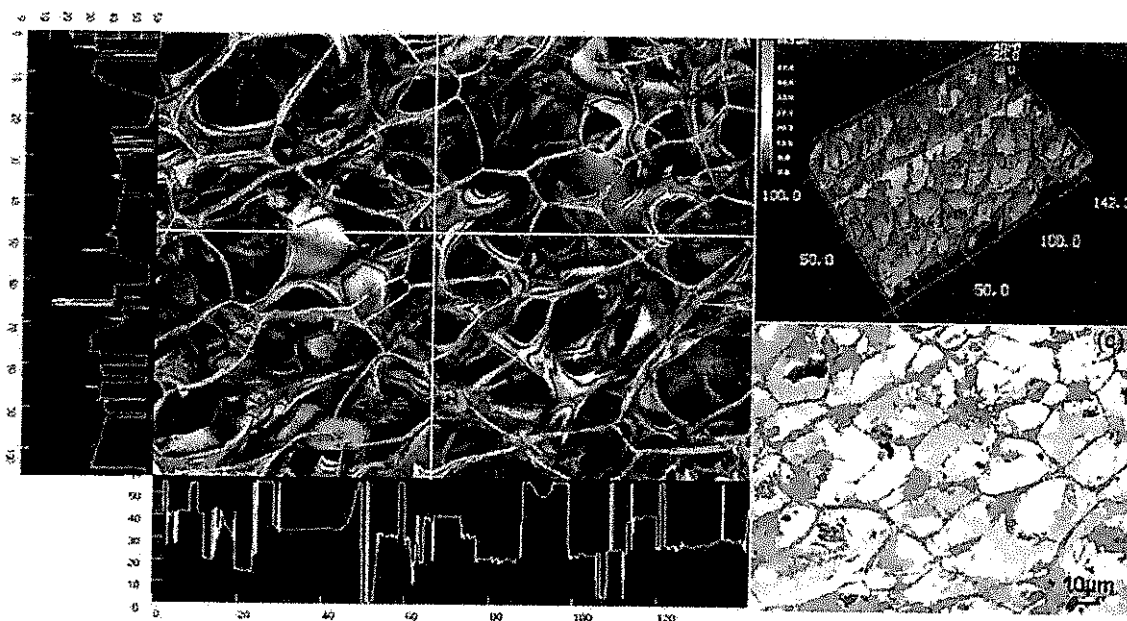


Image analysis of butyl-chitosan, scaffold 100x magnification, frozen -20C/ 1h, freeze dried

(a) Laser image with horizontal and vertical profile

(b) 3D height profile with horizontal cross section from (a)

(c) Height profile

Figure 4

Contribution to research and research-related education currently proposed to the DoD

The PI(s) are currently participating in the Hawaii Environmental and Energy Technology (HEET) initiative (PI: Richard Rocheleau) to support fuel cell technology development. Through the various projects supported by HEET funds, the Hawaii Natural Energy Institute is helping Navy and commercial developers such as UTC Powers to test and develop components and cells (i.e., characterizing membrane and membrane electrode assembly), all of which will benefit from pore structure information to be quantified. The Keyence microscope has already been introduced to researchers at HNEI and initial explorations have already begun.

Contribution to the establishment of new research capabilities

The PI is actively pursuing the characterization of the multidimensional and multidirectional pore structure of biocatalytic electrodes in terms of surface area that is both accessible to enzymes during the immobilization process as well as to liquid fuels transported through the pore structure. The preliminary images provided above give significant imaging into the degree of interconnectivity between pores, as well as the degree of multidimensional and multidirectionality.

Contribution to the enhancement of current research capabilities for performing research and research related education in areas of interest to the DoD

Electrochemical performance of enzyme-catalyzed surfaces benefits from correlation to the liquid phase mass transfer characteristics of the porous electrode. The permeability of enzyme-catalyzed electrodes, therefore, is an important measurement to characterize the charge transfer efficiency of enzyme catalyzed electrodes. When coupled with measurement of the total enzyme loading and its spatial distribution, a more comprehensive characterization of electrochemical performance is facilitated. The Keyence microscope images makes it possible to obtain SEM quality images of the pore structure, and within a software structure that permits assisted analysis of pore size and topographical surface roughness. The combination of topographical mapping with SEM quality imaging, on samples requiring no pretreatment, is quite a novel pore characterization technique. When combined with traditional measurements provided by mercury porosimetry, a far more complete characterization of pore structure can be obtained. In addition, we have the possibility to image the surface colonization of these structures with bacterial important to microbial fuel cells. In these systems these images are critical to evaluating the effect of colonization on the porosity and permeability of macroporous electrodes.

Contribution to existing facilities or upgrading of other instrumentation currently available for research and research-related education.

The Keyence microscope is currently being used by staff in the Hawaii Natural energy to analyze surface roughness of gold films, and the pore structure of charcoal. With regards to the surface roughness of gold films, the Keyence is being used as a secondary measurement of film thickness to help correlate thickness measurements made by the imaging ellipsometer in the laboratory of Dr. Bor Yann Liaw. With respect to the charcoal, these images are being used to

correlate to BET measurements of pore size, as well as SEM and wettability measurements in the laboratory of Dr. Michael Antal.

Special circumstances regarding the purchase or installation of the instrumentation.

None. The Keyence microscope is a bench top instrument that has been easily accommodated in the laboratory of the PI.